[CANCER RESEARCH 63, 776–779, February 15, 2003]

Advances in Brief

Enhancing the Efficacy of Photodynamic Cancer Therapy by Radicals from Plant Auxin (Indole-3-Acetic Acid)[1]

Lisa K. Folkes and Peter Wardman[2]

Cancer Research UK Free Radicals Research Group, Gray Cancer Institute, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, United Kingdom

Abstract

Indole-3-acetic acid (plant auxin) has low toxicity but dramatically enhances the killing of mammalian cells on illuminating phenothiazinium dyes with red light. Suitable dyes include toluidine blue, used in cancer diagnosis because of localization in tumors, and methylene blue, used in experimental photodynamic therapy of cancer. The photosensitized oxidation of indole acetic acid forms a free radical that fragments in microseconds, forming reactive cytotoxins. Unlike conventional photodynamic therapy, requiring excitation of oxygen to the reactive singlet state, the treatment is effective even at the low oxygen levels common in tumors and with much lower light doses than normally used.

Introduction

Treatment of cancer by PDT[3] usually involves the generation of reactive, excited oxygen molecules (1). A photosensitizing dye absorbs light, exciting it to an energetic triplet state capable of transferring its energy efficiently to ground state, triplet molecular oxygen to form excited, singlet oxygen. The latter is the damaging species, but its efficiency of production is much reduced at low oxygen tensions. Tumors are often poorly vascularized and have hypoxic regions; furthermore, oxygen depletion in the treatment area during PDT is a problem (2). Because red light penetrates tissue much deeper than that of shorter wavelength, photosensitizers absorbing in the red are preferred. Phenothiazinium dyes such as MB^+ or TB^+ absorb strongly at approximately 600–670 nm, and have been identified as having potential for cancer diagnosis or treatment. TB^+ has been widely evaluated as a diagnostic agent for oral cancers because of tumor localization (*e.g.*, OraScreen and OraTest; Zila Pharmaceuticals Inc.; Ref. 3), and a recent study (4) has assessed *in vitro* its potential for PDT. MB^+ has been extensively investigated as a photocatalyst of oxidative processes (5), in experimental PDT *in vitro* (6) and *in vivo* (7), and in limited clinical studies involving PDT (8, 9). We hypothesized that these dyes could be photocatalysts of oxidation of IAA, the commonest plant auxin (growth hormone), the dye triplet state oxidizing IAA to produce a radical-cation (Fig. 1). It is well known that some peroxidases oxidize IAA via a free-radical pathway, and the fast fragmentation of the radical-cation has been characterized (10). We have shown that products of IAA oxidation are cytotoxic to mammalian cells, including human tumor cells (11, 12). Thus, the combination of IAA or suitable analogues with these photosensitizers could provide an alternative route to photosensitized cell killing not involving singlet oxygen. Fig. 1 shows the conventional "type II" activation of oxygen to its singlet state, along with the main pathways established after oxidation of IAA to its radical-cation in a "type I" process, and a simplified scheme for the "redox cycling" or reoxidation of the dye free radical back to the active dye. We show here that IAA, a common, nontoxic plant chemical, is transformed into potent cytotoxins using red light and these phenothiazinium dyes that are already in clinical use. Activating IAA rather than oxygen to form cytotoxins offers the potential to treat hypoxic tumors effectively.

Materials and Methods

Reagents. IAA, MB^+ (recrystallized from methanol/benzene), and TB^+ were from Sigma. PBS [PBS, 0.14 M NaCl, 3 mM KCl, and 10 mM phosphate (pH 7.4)] was from Oxoid Ltd. (Basingstoke, Hampshire, United Kingdom). Sources of other chemicals and media have been described (11–13).

Cells. Chinese hamster V79 lung fibroblast cells and MCF7 human breast carcinoma cells were obtained from the European Collection of Cell Cultures and cultured as described previously (13).

Phototoxicity Measurements. Cells (200–20, 000) were plated into standard six-well plates and allowed to attach for 1 h (V79) or 4 h (MCF7) before replacing the medium with IAA (100 μM) and photosensitizer (2 μM) in PBS (2 ml, ~2.1 mm solution depth). The light source for 630-nm illumination was an 8 × 7 cm matrix of 200 LEDs (Agilent HLMP-ED18-TW000; Farnell Electronic Components, Leeds, United Kingdom) positioned ~13 cm above the six-well plate, which was placed over a mirror to reflect light. For 660-nm illumination, four adjacent 5-cm diameter circular clusters of 50 LEDs (Kingbright BL0307-50-44; RS Components Ltd., Corby, Northamptonshire, United Kingdom) were placed ~11 cm above the six-well plate. The illumination intensities were estimated using a DUO power meter (Gentec Electro-Optics; Quebec City, Canada) with PS-310 detector. Cells with MB^+ were illuminated at ~660 nm, intensity ~4.6 mW/cm^2; cells with TB^+, ~630 nm, intensity ~13.3 mW/cm^2. Illumination began immediately after adding IAA and photosensitizer, except for the experiments involving hypoxia, which involved 15 min of preincubation with frequent, gentle agitation for gas equilibration with 1% v/v O_2, 94% N_2, and 5% CO_2 (British Oxygen Co., Guildford, United Kingdom). Separate measurements using an OxyLite oxygen probe (Oxford Optronix Ltd., Oxford, United Kingdom) showed that equilibration with 1% oxygen was achieved.

After various illumination times, cells were removed from light, held at 37°C for 1 h, and the buffer was removed and replaced with 3 ml of culture medium without IAA or photosensitizer. The cells were then left to grow for 6 days (V79) or 10 days (MCF7) before fixing the colonies, staining, and counting as described previously (13).

Measurements of Photoproducts. Analyses of IAA and oxidation products involved HPLC with a RPB 100 × 3.2 mm column (Hichrom, Reading, United Kingdom) eluting with ammonium acetate [50 mM (pH 5.1)] at 1 ml/min with a linear gradient of 0–60% methanol over 8 min, using a photodiode array detector (Waters 996; Elstree, Hertfordshire, United Kingdom).

Results

The hypothesis was tested using mammalian cells *in vitro*; chemical products were measured in cell-free systems. Light doses of 1–6 J/cm^2 (a few minutes of illumination with a matrix of red LEDs) were sufficient to kill >99% of either Chinese hamster V79 lung fibroblast cells or MCF7 human breast carcinoma cells, using a concentration of

Received 9/20/02; accepted 12/27/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by Cancer Research UK Programme Grant C134/A2001.

[2] To whom requests for reprints should be addressed, at Gray Cancer Institute, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, United Kingdom. E-mail: wardman@gci.ac.uk.

[3] The abbreviations used are: PDT, photodynamic therapy; MB^+, methylene blue; TB^+, toluidine blue; IAA, indole-3-acetic acid; LED, light-emitting diode; MOI, 3-methylene-2-oxindole; HPLC, high-performance liquid chromatography.

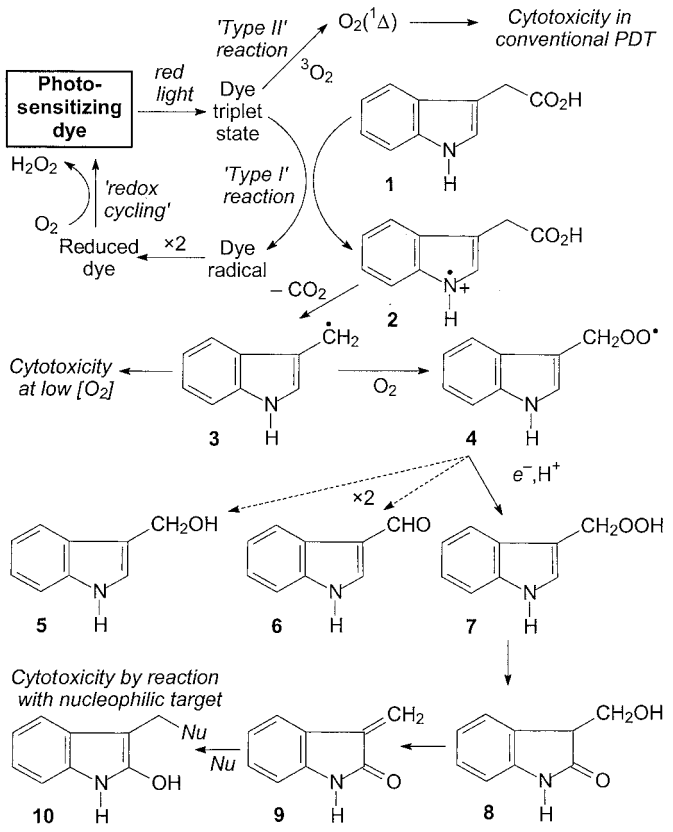

Fig. 1. Pathways involved in the photosensitized formation of MOI (*9*) from IAA (*1*). The triplet state of the dye reacts with IAA, forming the radical-cation 2, which fragments in ~40 μs to form the skatolyl radical 3; this may contribute to cytotoxicity at low oxygen levels, but it normally adds oxygen yielding the peroxyl radical 4. This disproportionates to form the alcohol 5 and the aldehyde 6, as well as forming the hydroperoxide 7. The latter breaks down via 8 to form the oxindole 9, which is reactive toward cellular nucleophiles (*Nu*), forming adducts 10.

IAA likely to be achievable in humans (see below). Fig. 2*A* shows that MB^+ without IAA was hardly phototoxic at these very low light doses, but the presence of IAA enhanced cell kill dramatically. The dyes alone, without illumination, or IAA alone (11, 13) have no detectable toxicity at these concentrations and exposure times. Similar experiments using TB^+ as photosensitizer required about twice the illumination for similar cell kill (Fig. 2*B*). However, from the measured absorbances of the solutions, with two light passes through ~2.1 mm of medium, it is calculated that ~12% of the light was absorbed with 2 μM MB^+ and ~6% with TB^+. Hence, the efficiencies of the two dyes were broadly similar. (Thus, the absorbed light doses in the present experiments are of the order of one hundredth of the doses commonly used in PDT, which are up to ~100 J/cm^2; Refs. 1, 14). Fig. 2, *C* and *D*, shows that human breast carcinoma MCF7 cells were more sensitive to the treatment than V79 cells, with about half the illumination times needed for comparable cell kill.

The laboratory environment is not representative of tumors; in particular, the oxygen concentration in air-equilibrated medium is ~20-fold higher than many tumor cells. Experiments were extended to V79 cells at 37°C equilibrated with 1% oxygen, a level common in tumors. Fig. 2*E* shows that phototoxicity of TB^+ with IAA in PBS at 37°C equilibrated with air required shorter illumination times for toxicity comparable with the experiments at room temperature (Fig. 2*B*), probably resulting in part from increased intracellular uptake of the photosensitizer during the 15-min preillumination incubation used. Fig. 2*F* shows that reducing the oxygen level from 21% (air; Fig. 2*E*) to 1% v/v did not have a marked effect on the phototoxicity of the TB^+/IAA combination. However, cells subjected to illumination in hypoxia grew into very small, diffuse colonies, suggestive of different or additional mechanism(s) of cytotoxicity compared with aerobic treatment.

Illumination of cell-free phosphate-buffered solutions of IAA and

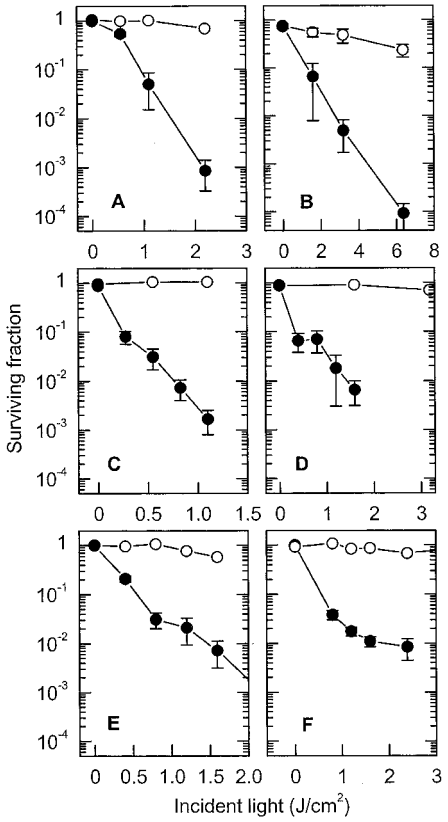

Fig. 2. Clonogenic cell survival after illumination of cells *in vitro* in the presence of 2 μM dyes without IAA (○) or with 100 μM IAA (●). *A–D*, cells illuminated in air at room temperature. *A*, V79 cells, MB^+. *B*, V79 cells, TB^+. *C*, MCF7 cells, MB^+. *D*, MCF7 cells, TB^+. *E*, V79 cells, TB^+, air, 37°C with 15 min preincubation before illumination commenced. *F*, V79 cells, TB^+, 1% oxygen, 37°C with 15 min preincubation before illumination. Data reflect means of three independent experiments (*error bars* not shown where smaller than symbol size); *bars*, ±SE.

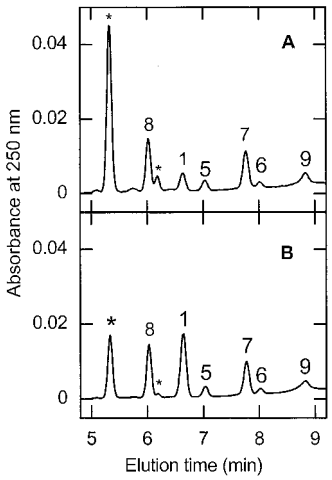

Fig. 3. Measurement of products from photosensitized decomposition of IAA. HPLC chromatograms after illumination (12 min) of 100 μM IAA with 2 μM MB^+ (*A*) or TB^+ (*B*) in PBS, using the same light sources, geometry, vessels, and volume of solutions as in the cell work. *Peak labels* correspond to the structures labeled in Fig. 1; those asterisked (*) may result from reaction of IAA with singlet oxygen.

Table 1 *Concentrations of IAA and quantifiable products after illumination of IAA (100 μM) and TB^+ (2 μM) in PBS with 630 nm light for 4 min at ~20 mW/cm² in a cuvette (1-cm path) at room temperature*

	IAA (1)[a] (μM)	Alcohol (5)[a] (μM)	Aldehyde (6)[a] (μM)	Hydroperoxide (7)[a,b]	Oxindole (8)[a,b]	MOI (9)[a] (μM)
Air	58 ± 3	5.4 ± 0.1	4.0 ± 0.6	1.00[b]	1.00[b]	0.4 ± 0.1
Air + 1 h at 37°C	57 ± 3	4.5 ± 0.3	1.3 ± 0.2	0	2.7 ± 0.5[b]	4.7 ± 0.3
1% O_2	87 ± 1[c]	3.1 ± 0.1[d]	1.2 ± 0.1[c]	0.37 ± 0.14[b]	0.8 ± 0.3[b]	0.23 ± 0.02
1% O_2 + 1 h at 37°C	87 ± 3[c]	2.8 ± 0.1[c]	0.17 ± 0.03[c]	0	1.4 ± 0.2[b,c]	2.4 ± 0.4[c]

[a] Products identified in parentheses as in Fig. 1; mean ± SE from two experiments.
[b] Relative concentrations from HPLC peak areas (normalized to illumination in air treatment) because calibration material was not available.
[c] Significantly different from the value obtained using air-saturated solutions; $P < 0.05$.
[d] Significantly different from the value obtained using air-saturated solutions; $P < 0.01$.

MB^+ or TB^+ showed that MOI (Fig. 1, *9*) and a number of precursors were produced (Fig. 3). Table 1 shows that the yield of MOI increased considerably on standing at 37°C for 1 h, together with complete loss of the hydroperoxide precursor (Fig. 1, *7*) and an increase in the amount of the intermediate (Fig. 1, *8*). The concentration of the aldehyde (Fig. 1, *6*) also decreased on standing at 37°C. Bubbling the solutions with 1% oxygen reduced both the yield of MOI and the loss of IAA. Lower IAA destruction probably arises from much-reduced singlet oxygen production, which degrades IAA to other products; the HPLC peaks thought to result from the attack of singlet oxygen on the indole ring were reduced ~50-fold in 1% oxygen compared with air.

To help assess whether the concentrations of IAA (100 μM) used in these studies *in vitro* might be achieved in tumors, tissue samples in mice bearing the mammary carcinoma NT were analyzed 30 min after administration of IAA (50 mg/kg i.p., 5 mg/ml in 50 mM $NaHCO_3$/2% v/v ethanol/water). The concentrations of IAA in tumor, plasma, and liver were 306 ± 64, 885 ± 93, and 675 ± 46 μM, respectively (mean ± SE; $n = 3$). No evidence of toxicity was observed.

Discussion

The results validate the hypothesis that photosensitized oxidation of IAA by phenothiazinium dyes and red light offers the potential to kill oxic and hypoxic tumor cells using low light doses. Much relevant data already exists to facilitate rational and rapid development of this concept. IAA is a catabolite of tryptophan, with micromolar levels in human plasma. Doses of IAA of 100 mg/kg p.o. were given to 18 humans in an early study for another purpose (15), with some reduction in blood glucose levels but no other effects reported. In another clinical investigation (16), doses of IAA corresponding to approximately 40–150 mg/kg p.o. were given, also with apparently no untoward effects. Only 50 mg/kg IAA administered to mice in the present work was sufficient to achieve tumor concentrations of IAA three times those used in the *in vitro* experiments. The phenothiazinium dyes have already been used clinically in the context of cancer therapy with topical (3) or oral administration (8), infusion into the bladder (8), and intravenous (17) or intratumor (9) injection. MB^+ is used as a treatment of methemoglobinemia at 1–2 mg/kg i.v., and its pharmacokinetics in blood and urine in humans after i.v. and oral dosage (100 mg; approximately 4–5 μmol/kg) have been studied, as well as organ distribution in rats (18). There is interest in optimizing the biodistribution properties of similar dyes (6). An increasingly important medical use of MB^+ exploiting its photodynamic activity is to reduce viral contamination of blood plasma by MB^+ and light. Other clinical uses of MB^+ include treatment of ifosfamide-induced encephalopathy (17) and methemoglobinemia. The recent study of PDT with TB^+ *in vitro* (4) noted the safety data available on the use of TB^+ in humans. Thus, whereas a full toxicological investigation is required, there is every expectation that tumor levels of IAA and of the photosensitizer comparable with those used in the present study could be achieved in humans.

Broadly similar effects in both V79 fibroblasts and MCF7 tumor cells were expected: earlier work involving peroxidase-catalyzed oxidation of IAA or a fluorinated analogue had shown that the treatment was effective in a variety of tumor cells (13, 19), although the cytotoxic response might vary with the levels of nucleophiles competing with the critical target(s) for the reactive methylene oxindole. The present experiments cannot replicate quantitatively the potential application of photosensitized formation of cytotoxins from IAA *in vivo*. The majority of the incident light was absorbed by the extracellular medium rather than the monolayers of attached cells. Other studies (4, 6) involving MB^+ or TB^+ *in vitro* have used loading of the cells with the dyes (which accumulate intracellularly) for up to several hours followed by removal of the extracellular dye before illumination. The calculation of absorbed light doses is difficult in such experiments. In the present work, limited measurements were made where attached V79 cells were incubated with TB^+ (10 μM) with or without IAA (100 μM) for 30 min, followed by washing with PBS and illuminating in fresh PBS containing neither dye nor IAA. As expected, cell kill required light doses considerably higher compared with the experiments when extracellular dye absorbed much more light. However, the surviving fraction of the cells that had been pretreated with IAA was ~5-fold lower than those treated with TB^+ alone. The intracellular concentration of IAA is difficult to assess in such experiments and is likely to be much reduced after washing.

A problem with conventional PDT is the absolute requirement for oxygen and reduced efficacy at low oxygen tension (2, 14). The IAA photo-oxidation can use oxygen, but the addition of oxygen to the skatolyl radical (Fig. 1, structure 3) is extremely fast, so that only very low levels would be needed. The very low light doses used in the present study suggest that a high light dose rate (intensity) would be unnecessary, reducing the possibility of local oxygen depletion, and low oxygen concentrations should be sufficient to ensure dye redox cycling. Furthermore, experiments using anaerobic peroxidase-catalyzed oxidation of IAA via gene transfection had shown cytotoxicity, although reduced compared with aerobic experiments (19). Cytotoxicity in anoxia or hypoxia may arise via reactions of the skatolyl radical. The cytotoxicity of methylene oxindole and reactivity toward thiols as illustrative nucleophiles were reported recently (12). Methylene oxindole is produced on illumination of IAA in the presence of riboflavin or Rose Bengal (20). It is possible that the cytotoxicity of photoproducts of riboflavin and IAA (21) involves in part similar pathways as shown in Fig. 1, although the authors found similar effects with tryptophan and tyrosine, suggesting different mechanisms.

In conclusion, the present results show that the phototoxicity of MB^+ and TB^+ is enhanced dramatically by the presence of IAA at concentrations likely to be achievable in humans. The use of IAA, or analogues with modified redox properties, with these or other suitable photosensitizers is attractive because of the opportunity for rational selection of both photosensitizer and IAA analogue, based on thermodynamic (redox) and kinetic arguments. In principle, a requirement is that the reduction potential of the photosensitizer triplet state [*e.g.*,

of the couple $^3MB^+/MB^\cdot$, where $MB^\cdot$ is the radical (semiquinone)] is higher than that of the indolyl radical cation ($IAA^{\cdot+}/IAA$); ideally, the reduced dye should also be efficiently "redox cycled" to the parent dye by oxygen. These requirements appear to be fulfilled for MB^+ and IAA. Rational consideration of alternative photosensitizers should be possible from kinetic studies and measurements of IAA-derived cytotoxins in cell-free systems.

Acknowledgments

We thank R. G. Newman and B. Vojnovic for construction of the light sources, S. Rossiter for supply of methylene oxindole, and M. R. L. Stratford for assistance with the measurements of distribution of IAA in animal tissues.

References

1. Dougherty, T. J., Gomer, C. J., Henderson, B. W., Jori, G., Kessel, D., Korbelik, M., Moan, J., and Peng, Q. Photodynamic therapy. J. Natl. Cancer Inst., *90:* 889–905, 1998.
2. Busch, T. M., Hahn, S. M., Evans, S. M., and Koch, C. J. Depletion of tumour oxygenation during photodynamic therapy: detection by the hypoxia marker EF3 [2-(2-nitroimidazol-1[*H*]-yl)-*N*-(3, 3, 3-trifluoropropy)acetamide]. Cancer Res., *60:* 2636–2642, 2000.
3. Onofre, M. A., Sposto, M. R., and Navarro, C. M. Reliability of toluidine blue application in the detection of oral epithelial dysplasia and *in situ* and invasive squamous cell carcinomas. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endol., *91:* 535–540, 2001.
4. Tremblay, J-F., Dussault, S., Viau, G., Gad, F., Boushira, M., and Bissonnette, R. Photodynamic therapy with toluidine blue in Jurkat cells: cytotoxicity, subcellular localization and apoptosis induction. Photochem. Photobiol. Sci., *1:* 852–856, 2002.
5. Tuite, E. M., and Kelly, J. M. Photochemical interactions of methylene blue and analogues with DNA and other biological substrates. J. Photochem. Photobiol. B, *21:* 103–124, 1993.
6. Mellish, K. J., Cox, R. D., Vernon, D. I., Griffiths, J., and Brown, S. B. *In vitro* photodynamic activity of a series of methylene blue analogues. Photochem. Photobiol., *75:* 392–397, 2002.
7. Orth, K., Beck, G., Genze, F., and Rück, A. Methylene blue mediated photodynamic therapy in experimental colorectal tumors in mice. J. Photochem. Photobiol. B, *57:* 186–192, 2000.
8. Williams, J. L., Stamp, J., Devonshire, R., and Fowler, G. J. S. Methylene blue and the photodynamic therapy of superficial bladder cancer. J. Photochem. Photobiol. B, *4:* 229–232, 1989.
9. Orth, K., Ruck, A., Stenescu, A., and Beger, H. G. Intraluminal treatment of inoperable oesophageal tumours by intralesional photodynamic therapy with methylene blue. Lancet, *345:* 519–520, 1995.
10. Candeias, L. P., Folkes, L. K., Dennis, M. F., Patel, K. B., Everett, S. A., Stratford, M. R. L., and Wardman, P. Free-radical intermediates and stable products in the oxidation of indole-3-acetic acid. J. Phys. Chem., *98:* 10131–10137, 1994.
11. Folkes, L. K., and Wardman, P. Oxidative activation of indole-3-acetic acids to cytotoxic species–a potential new role for plant auxins in cancer therapy. Biochem. Pharmacol., *61:* 129–136, 2001.
12. Folkes, L. K., Rossiter, S., and Wardman, P. Reactivity toward thiols and cytotoxicity of 3-methylene-2-oxindoles, cytotoxins from indole-3-acetic acids, on oxidation by peroxidases. Chem. Res. Toxicol., *15:* 877–882, 2002.
13. Folkes, L. K., Greco, O., Dachs, G. U., Stratford, M. R. L., and Wardman, P. 5-Fluoroindole-3-acetic acid: a prodrug activated by a peroxidase with potential for use in targeted cancer therapy. Biochem. Pharmacol., *63:* 265–272, 2002.
14. Stewart, F., Baas, P., and Star, W. What does photodynamic therapy have to offer radiation oncologists (or their cancer patients)? Radiother. Oncol., *48:* 233–248, 1998.
15. Mirsky, I. A., and Diengott, D. Hypoglycemic action of indole-3-acetic acid by mouth in patients with diabetes mellitus. Proc. Soc. Exp. Biol. Med., *93:* 109–110, 1956.
16. Rysánek, K., and Vítek, V. Increased excretion of 5-hydroxy-indole-acetic acid after administration of 3-indole-acetic acid (heteroauxine). Experientia, *15:* 217–218, 1959.
17. Pelgrims, J., De Vos, F., Van den Brande, J., Scrjvers, D., Prové, A., and Vermorken, J. B. Methylene blue in the treatment and prevention of ifosfamide-induced encelphalopathy: report of 12 cases and a review of the literature. Br. J. Cancer, *82:* 291–294, 2000.
18. Peter, C., Hongwan, D., Küpfer, A., and Lauterburg, B. H. Pharmacokinetics and organ distribution of intravenous and oral methylene blue. Eur. J. Clin. Pharmacol., *56:* 247–250, 2000.
19. Greco, O., Rossiter, S., Kanthou, C., Folkes, L. K., Wardman, P., Tozer, G. M., and Dachs, G. U. Horseradish peroxidase-mediated gene therapy: choice of prodrugs in oxic and anoxic tumor conditions. Mol. Cancer Ther., *1:* 151–160, 2001.
20. Amat-Guerri, F., Martínez-Utrilla, R., and López-González, M. M. C. Direct and dye-sensitized aqueous photo-oxidation of 3-indoleacetic acid, methyl-3-indoleacetate and 1-methyl-3-indoleacetic acid. I: Analysis of products. J. Photochem. Photobiol. A, *50:* 361–375, 1990.
21. Edwards, A. M., Barredo, F., De Ionnes, A. E., and Becker, M. I. Apoptosis induction in nonirradiated human HL-60 and murine NSO/2 tumor cells by photoproducts of indole-3-acetic acid and riboflavin. Photochem. Photobiol., *70:* 645–649, 1999.